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Virus Budding

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Overview

To spread infection, viruses must exit producer cells and transmit their genetic material into target cells. Viruses have evolved two general strategies for cellular egress: (1) Enveloped viruses acquire a host-derived lipid membrane as they breach the limiting membranes of the cell. During this process, the viral membrane typically also acquires one or more viral glycoproteins that bind target cell receptors and facilitate the membrane fusion step required for viral entry. (2) Non-enveloped viruses, by contrast, exit cells when the plasma membrane is disrupted, typically by cell lysis. Non-enveloped viruses then infect target cells either by disrupting limiting membranes to gain access to the cytoplasm or by pumping their nucleic acids directly into the cell. In recent years, the distinction between enveloped and non-enveloped viruses has been blurred by the discovery that some viruses, traditionally thought of as non-enveloped, can exit cells within vesicles. These are termed "quasi-enveloped" viruses.

The process of enveloped virus release comprises a series of coordinated steps, which are illustrated for human immunodeficiency virus type 1 (HIV-1) in **Fig. 1**:

(1) *Assembly*: Viral proteins and other essential components co-assemble to form virions. Many viruses assemble at the plasma membrane, but others assemble at internal membranes or in the cytoplasm before trafficking to the plasma membrane or exiting via the secretory system.
(2) *Envelopment*: The host membrane is bent and wrapped around the nascent virion.
(3) *Budding*: The membrane stalk connecting the virion to the host membrane is constricted and severed to release the enveloped particle.
(4) *Maturation*: Most enveloped viruses undergo further proteolytic and conformational maturation steps during or after budding. Maturation converts the assembly-competent virion into an infectious virus that can enter, uncoat, and replicate in the new target cell.

The complex series of events that accompany enveloped viral egress must be coordinated spatially and temporally, and these events are typically orchestrated by virally-encoded, multifunctional structural proteins. These proteins bind and remodel the membrane, self-assemble into virions, package other essential components such as nucleic acids into the nascent virion, and contain or recruit all of the activities necessary for budding and maturation. This article will describe general principles of enveloped virus assembly and release using the well-characterized HIV-1 Gag protein as a paradigm for a viral structural protein. Important principles employed by other viral families will also be discussed.

Assembly

All retroviruses, including HIV-1, express a Gag polyprotein that coordinates assembly of the immature virion (Sundquist and Krausslich, 2012; Meng and Lever, 2013; Lingappa *et al.*, 2014). When expressed alone, Gag is released within virus-like particles (VLPs), indicating that it contains or recruits all activities necessary for assembly, envelopment, and budding. An exciting new

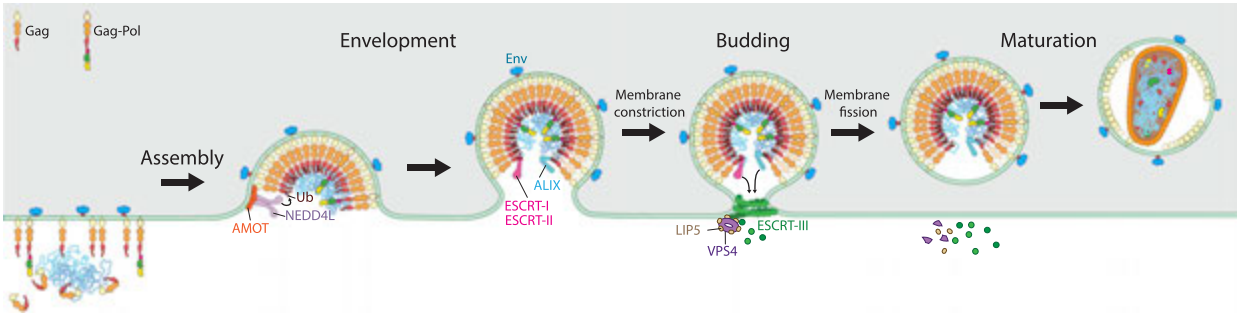

Fig. 1 *Stages of HIV-1 virion formation.* HIV-1 virion formation is coordinated by the multifunctional structural polyprotein Gag. Complexes of Gag with the viral RNA genome (blue) traffic to the plasma membrane where Gag assembles and binds other virion components, including the longer Gag-Pol polyprotein, which contains the viral protease, reverse transcriptase and RNase H enzymes. Gag also recruits three different host factors to facilitate membrane constriction and fission: AMOT-NEDD4L (orange and light purple, respectively), and the early-acting ESCRT-associated factors, ESCRT-I/II (pink) and ALIX (blue), which in turn recruit ESCRT-III proteins (green). ESCRT-III proteins form polymeric filaments that constrict the bud neck with the help of the VPS4 AAA+ ATPase and its cofactor LIP5. Protease activation during budding leads to Gag and Gag-Pol processing and formation of the mature, infectious virion.

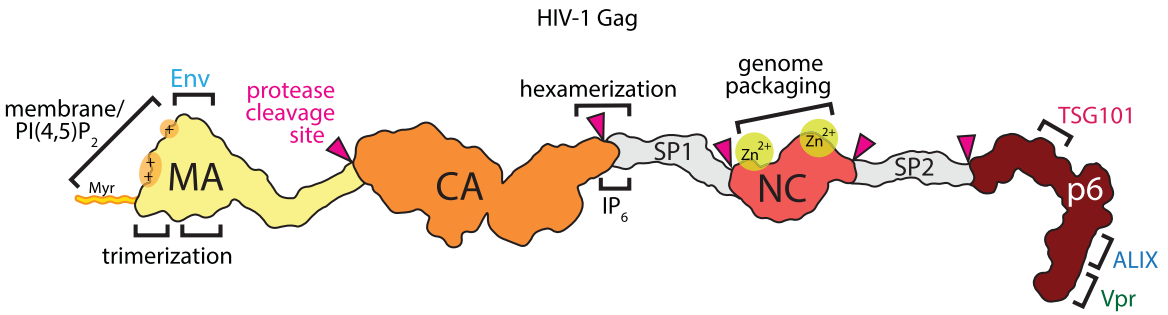

Fig. 2 *The HIV-1 Gag polyprotein.* HIV-1 Gag comprises four functional elements connected by two spacer peptides SP1 and SP2 (gray). MA (yellow) facilitates membrane binding and Env incorporation. CA (orange) mediates assembly of the immature capsid and, after proteolytic processing, forms the mature conical capsid. NC (red) binds the viral RNA genome through two zinc finger motifs. p6 (brown) binds Vpr and recruits early-acting ESCRT proteins TSG101 (a subunit of the ESCRT-I complex) and ALIX to facilitate membrane fission. Pink arrowheads denote proteolytic cleavage sites during maturation.

variation on this same theme is the discovery of Gag-related cellular proteins that can self-assemble, exit producer cells, and transfer RNA molecules between cells (Pastuzyn *et al.*, 2018; Ashley *et al.*, 2018).

As illustrated in **Fig. 2**, HIV-1 Gag comprises four functional elements: MA, CA, NC and p6, connected by two spacer peptides: SP1 and SP2. The MA domain in Gag (MA^{Gag}) targets assembly to the plasma membrane, NC^{Gag} binds the dimeric viral RNA genome, the CA^{Gag} and $SP1^{Gag}$ elements mediate spherical particle assembly, and $p6^{Gag}$ recruits cellular ESCRT (Endosomal Sorting Complexes Required for Transport) pathway factors required for virus budding. Gag also packages several other components required for full infectivity, including the viral enzymes, which are packaged as co-assembling Gag-Pol fusion proteins, the viral Env protein, whose intracellular domain interacts with MA^{Gag}, and Viral Protein R (Vpr), which binds $p6^{Gag}$.

(1) *RNA packaging*: Following synthesis in the cytoplasm, Gag monomers or low-order multimers bind two associated copies of the full-length viral genomic RNA through a highly structured packaging site located near the 5′ end of the genome (Jouvenet *et al.*, 2009; Kutluay and Bieniasz, 2010; Keane *et al.*, 2015). This interaction is mediated by NC^{Gag} and enables specific selection of the HIV-1 genome over cellular RNAs and viral RNAs undergoing translation (Kaddis Maldonado and Parent, 2016; Dubois *et al.*, 2018; Kuzembayeva *et al.*, 2014, Brown *et al.*, 2020). Several host proteins, including ABCE1, Staufen1, and DDX6, can associate with Gag-RNA complexes and have been proposed to promote Gag trafficking, multimerization, and/or genome encapsidation, although these activities are not yet fully defined (Reed *et al.*, 2012; Zimmerman *et al.*, 2002; Chatel-Chaix *et al.*, 2007).

(2) *Membrane trafficking*: Gag and Gag-RNA complexes move to the plasma membrane and associate with cholesterol-rich microdomains, commonly termed "lipid rafts" (Ono and Freed, 2001). Binding of Gag to the plasma membrane is mediated by a bipartite signal in the MA^{Gag} domain that comprises a post-translational myristic acid modification at the amino-terminus and a cluster of basic residues, the highly basic region (HBR). Prior to membrane binding, the myristate is sequestered in a hydrophobic cavity within the globular MA^{Gag} domain. The HBR interacts with acidic head groups of the plasma membrane-specific phospholipid phosphatidylinositol-(4,5)-bisphosphate ($PI(4,5)P_2$) and this interaction, perhaps in concert with Gag multimerization, exposes the myristate moiety. $PI(4,5)P_2$ and the conformational "myristoyl switch" enable targeting and stable Gag association with the plasma membrane (Chukkapalli and Ono, 2011; Tang *et al.*, 2004). In addition to $PI(4,5)P_2$, MA^{Gag} can also bind cellular RNA, especially tRNAs. The MA-tRNA interactions likely prevent non-specific binding of Gag to cellular membranes other than the plasma membrane (Chukkapalli *et al.*, 2010; Alfadhli *et al.*, 2011; Kutluay *et al.*, 2014; Gaines *et al.*, 2018).

(3) *Virion assembly*: At the plasma membrane, Gag molecules multimerize and assemble into a hexagonal lattice through lateral protein-protein interactions that are mediated by the CA-SP1 region (Schur *et al.*, 2016; Wagner *et al.*, 2016). Host-derived inositol hexakisphosphate (IP_6) binds within the CA-SP1 hexamers and facilitates formation of the immature Gag lattice (Dick *et al.*, 2018; Mallery *et al.*, 2018). In addition to the genomic RNA, Gag interacts directly with other viral proteins that are incorporated into the nascent virion, including the multifunctional accessory protein Vpr, which binds a leucine-rich element located within $p6^{Gag}$ (Kondo *et al.*, 1995), and the cytoplasmic tail of the gp41 subunit of the heterotrimeric transmembrane Env protein, which is incorporated within holes in the associating Gag lattice created by MA trimerization (Tedbury and Freed, 2014; Tedbury *et al.*, 2016; Hill *et al.*, 1996; Pezeshkian *et al.*, 2019). In addition to the Gag polyprotein, the full-length viral RNA also encodes the Gag-Pol polyprotein. The longer Gag-Pol protein is translated by a ribosomal frameshifting mechanism, contains the viral enzymes, and is incorporated into the nascent virion through interactions with Gag (Smith *et al.*, 1993).

Envelopment

The multifunctional structural proteins that mediate assembly and membrane targeting also appear to facilitate virion envelopment by inducing membrane curvature. The hexagonal HIV-1 Gag lattice contains small discontinuities that accommodate

Table 1 *Viral proteins that mediate assembly and budding of enveloped viruses.* Virus families and species are listed as described in the Virus Taxonomy: 2018b Release by the International Committee on Taxonomy of Viruses (ICTV). To be included in the table, virus families must be enveloped and contain at least one species that infects mammals

Virus family	*Example species*	*Protein(s) mediating assembly and budding*
Arenaviridae	Lassa virus, lymphocytic choriomeningitis virus	matrix protein Z
Arteriviridae	Equine arteritis virus, porcine reproductive and respiratory syndrome virus	envelope proteins GP5 and M
Asfarviridae	African swine fever virus	p72 (outer capsid layer), polyproteins pp220 and pp62 (inner core shell)
Bornaviridae	Mammalian 1 orthobornavirus	matrix protein M
Coronaviridae	Middle East respiratory syndrome-related coronavirus, severe acute respiratory syndrome-related coronavirus,	membrane protein M, envelope protein E
Filoviruses	Zaire ebolavirus, Marburg marburgvirus	matrix protein VP40
Flaviviridae	Dengue virus, West Nile virus, Yellow fever virus, hepatitis C virus	envelope proteins prM and E, aided by non-structural proteins
Hantaviridae	Sin Nombre orthohantavirus	envelope proteins Gn and Gc
Hepadnaviridae	Hepatitis B virus	envelope proteins S and L
Herpesviridae	human alphaherpesvirus	Nuclear egress complex (pUL31 and pUL34, primary envelopment), pUL7, pUL51 and other tegument proteins (secondary envelopment)
Matonaviridae	Rubella virus	envelope proteins E1 and E2
Nairoviridae	Crimean-Congo hemorrhagic fever orthonairovirus	envelope proteins Gn and Gc
Orthomyxoviridae	Influenza A virus, influenza B virus	matrix protein M1
Paramyxoviridae	Hendra henipavirus, Nipah henipavirus, measles morbillivirus	matrix protein M
Peribunyaviridae	Bunyamwera orthobunyavirus, La Crosse virus	envelope proteins Gn and Gc
Phenuiviridae	Rift Valley fever virus	envelope proteins Gn and Gc
Pneumoviridae	Human metapneumovirus, human orthopneumovirus	matrix protein M
Poxviridae	Vaccinia virus, variola virus	membrane proteins A14 and A17, scaffold protein D13 and other viral membrane assembly proteins (formation of mature virions); A27, B5, and F13 (formation of enveloped virions)
Rhabdoviridae	Rabies lyssavirus	matrix protein M
Retroviridae	Human immunodeficiency virus-1, human immunodeficieny virus-2,	Gag polyprotein
Tobaniviridae	Equine torovirus, porcine torovirus	membrane protein M
Togaviridae	Chikungunya virus, eastern equine encephalitis virus	envelope proteins E1 and E2

declination and allow the immature lattice to bend the membrane and create a spherical virion (Schur *et al.*, 2016). The host factor angiomotin (AMOT) has also been implicated in HIV-1 virion envelopment because fully enveloped spherical particles are not formed efficiently in the absence of AMOT (Mercenne *et al.*, 2015). The Bar domain of AMOT likely contributes to this activity as this domain has been shown to bend and tubulate membranes in other contexts (Nishimura *et al.*, 2018).

Unlike HIV-1, betaretroviruses and spumaviruses assemble in the cytoplasm before being trafficked to the plasma membrane. Thus, assembly and budding are spatially and temporally separated in these viruses. For example, Gag proteins from prototypical foamy virus, Mason-Pfizer monkey virus, and mouse mammary tumor virus preassemble into immature virions near the pericentriolar region and are then trafficked on microtubules to associate with membranes and the viral Env protein (Müllers, 2013; Hütter *et al.*, 2013; Swanstrom and Wills, 1997). Hepatitis B virus (HBV), a hepadnavirus, similarly forms capsids in the cytoplasm, which then associate with envelope proteins in cellular membranes to mediate particle envelopment and budding (Prange, 2012; Blondot *et al.*, 2016).

More broadly, most enveloped viruses have multifunctional structural proteins that mediate assembly and envelopment. Often, this role is fulfilled by matrix proteins that bind to the viral membrane, assemble into higher-order structures, and link internal ribonucleoprotein complexes to external envelope glycoproteins. In viruses that lack classical matrix proteins, such as hantaviruses (Hepojoki *et al.*, 2012; Cifuentes-Munoz *et al.*, 2014; Muyangwa *et al.*, 2015) and alphaviruses (Brown *et al.*, 2018), cytoplasmic tails of envelope glycoproteins act as matrix protein surrogates by directly interacting with nucleoproteins. Larger, more complex DNA viruses such as pox viruses (Roberts and Smith, 2008; Liu *et al.*, 2014) and herpes viruses (Lv *et al.*, 2019) divide these functions between multiple viral proteins (see **Table 1**).

Budding

ESCRT-dependent budding

As membrane envelopment proceeds, the membrane is constricted until nascent virions are connected to the plasma membrane by a thin stalk that must be severed to separate the viral and cellular membranes. Many enveloped viruses accomplish membrane constriction and fission by recruiting the machinery of the cellular ESCRT pathway (Votteler and Sundquist, 2013).

ESCRT-independent enveloped viruses also exist, however, and these viruses must therefore either recruit other, as yet unidentified, host factors or encode viral proteins that mediate budding.

The ESCRT pathway mediates cellular membrane fission events throughout eukarya and also in some archaeal species (McCullough *et al.*, 2018; Christ *et al.*, 2017; Scourfield and Martin-Serrano, 2017; Henne *et al.*, 2013). The pathway was initially identified as the machinery that mediates intraluminal vesicle budding into specialized late endosomes, termed multivesicular bodies (MVB) (Hanson and Cashikar, 2012), but is now known to act at many other cellular membranes, including during cytokinetic abscission (Carlton and Martin-Serrano, 2007; Morita *et al.*, 2007), resealing of the post-mitotic nuclear envelope (Olmos *et al.*, 2015; Vietri *et al.*, 2015), membrane repair (Scheffer *et al.*, 2014; Jimenez *et al.*, 2014; Skowyra *et al.*, 2018), closure of autophagosomes (Takahashi *et al.*, 2018; Zhou *et al.*, 2019), and neuronal pruning (Zhang *et al.*, 2014; Loncle *et al.*, 2015; Issman-Zecharya and Schuldiner, 2014). Notably, all of these membrane fission events involve constricting membranes toward a cytoplasm-filled neck and are therefore topologically equivalent to virus budding from the plasma membrane.

ESCRT-mediated membrane fission events are catalyzed by a common core machinery (McCullough *et al.*, 2018; Christ *et al.*, 2017; Scourfield and Martin-Serrano, 2017; Banjade *et al.*, 2019), which is recruited to different membranes by adapter proteins. These membrane-specific adapters recruit early-acting ESCRT proteins, which help to stabilize membrane curvature and also nucleate assembly of late-acting ESCRT-III proteins, which form the core fission machinery. ESCRT-III proteins can be recruited by three known mechanisms: (1) Adapters can recruit Bro1 domain-containing proteins such as ALIX, which serves as a bridge to the ESCRT-III proteins, (2) Adapters can bind the ESCRT-I complex, which in turn recruits ESCRT-III proteins via intermediate ESCRT-II complexes, and (3) The nuclear LEM2 adapter binds CHMP7, a hybrid ESCRT-II/ESCRT-III protein, which then binds other ESCRT-III proteins.

Humans express 12 related ESCRT-III proteins that are divided into eight subfamilies, CHMP1–7 and IST1, with some subfamilies comprising several homologs. ESCRT-III proteins can adopt "closed" and "open" conformations. In the autoinhibited closed state, ESCRT-III proteins are soluble and monomeric. When autoinhibition is relieved, ESCRT-III subunits open and can then bind membranes and form curved helical filaments. These filaments constrict membranes and recruit VPS4 AAA+ ATPases. VPS4 enzymes dynamically remodel and disassemble ESCRT-III filaments, using the energy of ATP hydrolysis to extract individual ESCRT-III subunits and release them back into the cytoplasm. These enzymes thereby power the virus budding cycle, although the precise mechanism by which ESCRT-III filaments and VPS4 enzymes collaborate to mediate fission is not yet fully understood.

Enveloped viral structural proteins recruit the ESCRT pathway using motifs that mimic cellular ESCRT adapters (Votteler and Sundquist, 2013; Hurley and Cada, 2018; Lippincott-Schwartz *et al.*, 2017). These motifs were initially discovered in retroviral Gag proteins (Gottlinger *et al.*, 1991; Huang *et al.*, 1995; Parent *et al.*, 1995; Xiang *et al.*, 1996) and were termed "late domains" because they exerted their effects at a late stage of assembly. Analogous late domains were subsequently identified in many other enveloped viruses. Late domains can frequently function from different positions within viral structural proteins and can be swapped between viruses (Parent *et al.*, 1995; Yuan *et al.*, 2000), consistent with the idea that although they have different primary binding partners, they all ultimately converge on common downstream ESCRT-III proteins. Several different classes of late domains are now well understood, and others have been identified but remain to be linked to ESCRT binding partners.

P(S/T)AP: The P(S/T)AP late domain (where the second residue can be either a serine or a threonine) was first identified in the p6 polypeptide of HIV-1 Gag (Gottlinger *et al.*, 1991; Huang *et al.*, 1995), and subsequently identified in structural proteins of other retroviruses, filoviruses, arenaviruses and reoviruses (Votteler and Sundquist, 2013). The P(S/T)AP motif recruits the four-protein ESCRT-I complex by binding the UEV domain of the TSG101 subunit (Demirov *et al.*, 2002; Garrus *et al.*, 2001; VerPlank *et al.*, 2001; Martin-Serrano *et al.*, 2001). P(S/T)AP motifs are found in several cellular ESCRT adapter proteins, such as the early endosomal protein HRS (Bache *et al.*, 2003).

YPX$_n$L: YPX$_n$L late domains (where X$_n$ can vary in sequence and length) recruit ALIX by binding the central V domain (Strack *et al.*, 2003; Martin-Serrano *et al.*, 2003; von Schwedler *et al.*, 2003). HIV-1 contains a YPX$_n$L late domain, although this motif is less critical for budding than the PTAP motif in most cell types. Other viruses rely exclusively or primarily on YPX$_n$L domains for budding, including other retroviruses, paramyxoviruses, flaviviruses, and possibly herpesviruses (Votteler and Sundquist, 2013). Divergent structural proteins that lack a readily detectable YPX$_n$L motif, yet still bind to ALIX, have also been described (Boonyaratanakornkit *et al.*, 2013; Lee *et al.*, 2012), suggesting that ALIX-recruiting sequence motifs may accommodate more variability than has been documented to date. Cellular YPX$_n$L-containing ESCRT adapters recruit ALIX during exosome biogenesis and lysosomal sorting (Baietti *et al.*, 2012; Dores *et al.*, 2012).

PPXY: The PPXY late domain (where X can be any residue but is often a proline) binds WW domains in NEDD4-like HECT ubiquitin E3 ligases. PPXY late domains are found in retroviruses, filoviruses, arenaviruses, rhabdoviruses, and hepadnaviruses (Votteler and Sundquist, 2013). HIV-1 Gag does not contain a recognized PPXY motif. Nevertheless, overexpression of the ubiquitin E3 ligase NEDD4L induces budding of HIV-1 Gag constructs that lack PTAP and YPX$_n$L late domains (Usami *et al.*, 2008; Chung *et al.*, 2008). In this context, NEDD4L is recruited by a cellular PPXY-containing protein AMOT, which also binds HIV-1 Gag (Mercenne *et al.*, 2015). The related angiomotin-like 1 (AMOTL-1) recruits NEDD4 family members to paramyxovirus M proteins, which also lack discernible PPXY domains (Pei *et al.*, 2010; Ray *et al.*, 2019). NEDD4 E3 enzymes transfer K63-linked ubiquitin chains onto PPXY-containing cellular proteins, and they regulate endocytosis, ESCRT-dependent MVB cargo selection and protein trafficking through ubiquitination (Rotin and Kumar, 2009).

It is not yet fully understood how NEDD4 family members promote budding, but it has been suggested that ubiquitination of viral structural proteins, or other proteins within the budding site, recruits the ESCRT pathway because ALIX and TSG101 both contain ubiquitin-binding domains that can recognize ubiquitinated cargos for MVB incorporation and lysosomal degradation

(Shields and Piper, 2011). Consistent with this idea, retroviral Gag proteins are typically ubiquitinated (although this requirement is not absolute (Zhadina *et al.*, 2007)), and ubiquitin depletion or mutations that prevent ubiquitin ligase recruitment inhibit retrovirus budding (Patnaik *et al.*, 2000; Strack *et al.*, 2000).

FPIV: The paramyxovirus SV5 employs an FPIV motif to facilitate budding. SV5 release is ESCRT-dependent and is augmented by AMOTL1, but the binding partner for the FPIV motif remains to be defined (El Najjar *et al.*, 2014).

Viral structural proteins typically encode multiple late domains that function in synergy. For example, HIV-1 $p6^{Gag}$ contains both a P(S/T)AP and a YPX_nL motif, the HTLV-I Gag and Ebola virus (EBOV) structural protein VP40 proteins contain adjacent PPPY and PTAP late domains that bind both TSG101 and NEDD4, and murine leukemia virus Gag contains all three canonical late domains (Votteler and Sundquist, 2013). Nevertheless, some viruses contain a single (or at least dominant), late domain. For example, the retroviral EIAV Gag protein appears to recruit ALIX exclusively through a single YPX_nL motif (Votteler and Sundquist, 2013).

All modes of viral ESCRT pathway recruitment ultimately converge on ESCRT-III, the machinery that catalyzes membrane fission. In the best-studied cases such as HIV-1, multiple different mammalian ESCRT-III proteins have been shown to localize to the bud neck (Jouvenet *et al.*, 2011), but only CHMP2 and CHMP4 family members seem to perform indispensable functional roles (Sandrin and Sundquist, 2013; Morita *et al.*, 2011). ESCRT-III proteins form helical filaments in the bud neck and progressively constrict it with the help of VPS4 enzymes, as described above. Ultimately, a membrane fission reaction severs the neck, releasing the virion from the cell.

ESCRT-independent budding

Some enveloped viruses bud independently of the ESCRT pathway, including alphaviruses (Brown *et al.*, 2018), some paramyxoviruses (Salditt *et al.*, 2010; Utley *et al.*, 2008), and influenza A virus (Rossman and Lamb, 2011). ESCRT-independent membrane scission mechanisms are generally not well understood, but appear to involve as yet unidentified cellular factors, virally encoded proteins, or a combination.

Some RNA viruses, such as alphaviruses, contain an outer glycoprotein shell that completely covers the exterior of the viral membrane. The formation of this external protein coat has been suggested to play a role in virus budding, both in ESCRT-independent and ESCRT-dependent viruses. The alphaviral transmembrane glycoproteins E1 and E2 are embedded in the viral lipid envelope and form heterodimers that further trimerize into a continuous icosahedral lattice. These interactions are required to complete the budding step, and completion of the E1-E2 protein lattice, together with the nucleocapsid interactions across the viral membrane may be sufficient to drive both membrane curvature and fission (Brown *et al.*, 2018; Weissenhorn *et al.*, 2013). In contrast, flaviviruses and hepaciviruses also contain an outer protein coat, but still depend on the ESCRT pathway to complete their replication cycle (see below). Thus, external protein coats can apparently either replace or act in concert with the ESCRT pathway.

Influenza A is another ESCRT-independent virus (Bruce *et al.*, 2009; Watanabe and Lamb, 2010; Chen *et al.*, 2007), but in this case, the viral transmembrane protein M2 appears to mediate membrane fission (Rossman *et al.*, 2010). During particle assembly, the hemagglutinin (HA) and neuraminidase (NA) glycoproteins are targeted to the plasma membrane and cluster in lipid rafts. The matrix protein M1 interacts with the cytoplasmic tails of HA and NA, polymerizes against the membrane, and apparently acts in concert with HA to induce membrane curvature. M1 also recruits M2 to the bud neck, and an amphipathic helix in the cytoplasmic M2 tail inserts, deforms and promotes plasma membrane fission (Martyna and Rossman, 2014; Chlanda and Zimmerberg, 2016). M2 also functions as a pH-regulated ion channel that facilitates the release of viral ribonucleoprotein complexes from the endosome into the cytoplasm, but ion channel and membrane fission appear to be independent activities (Rossman *et al.*, 2010).

Intracellular budding

Some viral families envelop and bud into internal cellular membranes rather than at the plasma membrane. Budding into the lumen of a cellular organelle is topologically equivalent to budding from the plasma membrane, but in these cases the viral particle is temporarily surrounded by two membranes, the viral envelope and the organelle membrane. Virion release into the extracellular space therefore requires transport to and fusion of the virion-containing organelle with the plasma membrane.

One such case is the hepatitis B virus (HBV), which co-opts the MVB pathway for egress (Prange, 2012; Blondot *et al.*, 2016; Patient *et al.*, 2009). The three viral envelope proteins S, M, and L form budding sites at the MVB membrane that recruit mature cytoplasmic nucleocapsids. The assembling HBV particles bud into the MVB lumen in an ESCRT-dependent reaction that creates intraluminal virions. MVBs then fuse with the plasma membrane to release the enveloped viral particles from the cell, a process that resembles exosome release.

Herpesviruses are released via the secretory pathway in a complex series of events that requires several viral and cellular proteins (Lv *et al.*, 2019; Fradkin and Budnik, 2016; Owen *et al.*, 2015). Herpesviral genome replication, capsid assembly, and genome packaging all take place in the nucleus. The fully assembled nucleocapsids are too large to escape into the cytoplasm through nuclear pores and instead exit the nucleus and cell by undergoing several steps of envelopment and de-envelopment at multiple cellular membranes. During primary envelopment, the nucleocapsids bud through the inner nuclear membrane into the perinuclear space, thereby acquiring a lipid envelope. This process requires the virus to remodel the nuclear lamina. Cellular and viral kinases phosphorylate components of the nuclear lamina, leading to its disassembly. Two viral proteins, pUL31 and pUL34, then assemble into a cage-like nuclear egress complex, that carries the virion across the inner nuclear membrane (Bigalke and Heldwein, 2015). There is some evidence that the ESCRT machinery may also be involved in facilitating the membrane fission step required

to release the enveloped virion into the intermembrane space (Arii *et al.*, 2018). The primary envelope then fuses with the outer nuclear membrane in a process termed de-envelopment. The viral glycoproteins are necessary for de-envelopment, likely because they mediate fusion with the outer nuclear membrane. Once in the cytoplasm, nucleocapsids associate with tegument proteins, which in the mature virion occupy the space between the nucleocapsid and the envelope. The nucleocapsids then bud into vesicles, whose origins have been variously described as the trans-Golgi network, endosomes, or autophagic membranes. Recruitment to sites of secondary envelopment is promoted by tegument proteins through interactions with vesicle membranes and viral glycoproteins. Early-acting ESCRT proteins are not required for this process, but there are reports that ESCRT-III and VPS4 activity are required for virion release (Crump *et al.*, 2007; Calistri *et al.*, 2007; Pawliczek and Crump, 2009), and an exciting new structure of the herpes simplex virus pUL7: pUL51 complex, which is required for efficient virion assembly, reveals that the N-terminal region of pUL51 adopts a CHMP4B-like fold that may function as a viral ESCRT-III-like protein (Butt *et al.*, 2020). Following membrane fission, the enveloped virions end up inside intracellular vesicles and are released into the extracellular space when the vesicles fuse with the plasma membrane.

In a related process, flaviviruses and hepaciviruses are released through the secretory pathway (Chatel-Chaix and Bartenschlager, 2014; Falcon *et al.*, 2017; Gerold *et al.*, 2017). Both genera of viruses induce extensive remodeling of endoplasmic reticulum (ER) membranes to form replication compartments. These vesicle-like structures remain connected to the ER, enclose viral proteins and the viral genome, and serve as protected compartments where almost all steps of the life cycle are carried out. Assembled nucleocapsids then bud into the ER lumen and are released through the secretory pathway.

Quasi-enveloped viruses

Historically, viruses have been divided into enveloped and non-enveloped classes based on the presence or absence of a host-derived membrane envelope, and it was thought that non-enveloped viruses were released exclusively by host cell lysis. This simple paradigm has now been overturned by studies showing that many different classes of non-enveloped viruses can acquire host-derived lipid envelopes and exit cells within vesicles. These extracellular vesicles resemble exosomes, and viruses that use this egress method are termed "quasi-enveloped" (Feng *et al.*, 2014).

The picornavirus hepatitis A virus (HAV) was the first virus definitively shown to be quasi-enveloped (Feng *et al.*, 2013), and HAV still serves as a paradigm for the process. Quasi-enveloped HAV particles (eHAV) are released in exosome-like vesicles that typically contain 1–4 particles per vesicle. These vesicles are formed when HAV capsids bud into endosomes in an ESCRT-dependent manner. To promote budding, the VP2 capsid protein recruits ALIX, apparently using tandem YPX_3L domains that become buried in the fully assembled virion (Gonzalez-Lopez *et al.*, 2018; McKnight *et al.*, 2017). Virion-containing multivesicular bodies then fuse with the plasma membrane and release eHAV particles into the extracellular space. There is now good evidence that this is the primary mode of HAV release from hepatocytes in vivo, and that HAV circulates in the blood exclusively within small vesicles (Feng *et al.*, 2013). HAV can then shed its envelope in the biliary tract, which produces a non-enveloped particle that may be more stable in harsher environmental conditions (Feng *et al.*, 2014). Importantly, HAV is highly infectious in both its enveloped and non-enveloped states.

The capacity for quasi-envelopment has since been described for several other viruses that were traditionally considered to be non-enveloped, including many other picornaviruses (Chen *et al.*, 2015; Mutsafi and Altan-Bonnet, 2018), Hepatitis E virus (Qi *et al.*, 2015), rotaviruses and noroviruses (Santiana *et al.*, 2018). Furthermore, some picornaviruses, including poliovirus and coxsackievirus, differ from the HAV paradigm in that they form quasi-enveloped virions by subverting the autophagy pathway. In these cases, double-membraned autophagosomes engulf multiple naked viral particles, which then release quasi-enveloped viruses when the outer autophagosomal membrane fuses with the plasma membrane (Mutsafi and Altan-Bonnet, 2018; Bird *et al.*, 2014). A final variation on this theme is the exosomal transfer of viral nucleic acids between cells, which apparently can, in some viruses like hepatitis C, spread productive infections without requiring full viral assembly (Ramakrishnaiah *et al.*, 2013; Bukong *et al.*, 2014).

The membrane appears to perform several important functions for quasi-enveloped viruses, including protecting the capsid from antibody-mediated neutralization (Feng *et al.*, 2013), and clustering together of multiple virions so that they can enter target cells as a swarm or "quasi-species" that can cooperate genetically through cross-complementation. The later activity may be most important for enteroviruses, whose larger vesicles can each contain tens or even hundreds of viral particles (Chen *et al.*, 2015; Santiana *et al.*, 2018).

The outer membranes of quasi-enveloped viruses lack viral glycoproteins, and therefore cannot fuse with target cell membranes. Instead, eHAV particles are taken up into the host cells by endocytosis and trafficked toward the lysosome, where the membrane is degraded, and the released naked virions can cross into the cytoplasm by disrupting the endolysosomal membrane (Rivera-Serrano *et al.*, 2019).

Maturation

Most enveloped viruses undergo additional maturation steps during and after budding. Before maturation, the virion functions as an assembly machinery that can package components and leave the producer cell. Conformational changes, typically triggered by proteolytic cleavage or pH changes, then convert the virion into a particle that is capable of entering and replicating in a new target cell (Veesler and Johnson, 2012; Steven *et al.*, 2005).

In the case of HIV-1, the viral protease (PR) is activated by autoproteolysis as the virus assembles and buds, and it cleaves the Gag polyprotein at five different sites, producing three new proteins (MA, CA and NC) and three smaller peptides (SP1, SP2, and p6). Gag processing drives a series of major rearrangements in which the CA protein forms a conical internal capsid that surrounds viral RNA in complex with NC protein and viral enzymes. Gag cleavage is a sequential, ordered process, and each processing event appears to perform a different function. Cleavage at the SP1-NC junction releases the NC-RNA complex to condense to the center of the virion, cleavage at the MA-CA junction promotes folding of the CA N-terminus into a β-hairpin that will ultimately form an NTP-permeable pore in the assembled capsid, and cleavage at the CA-SP1 junction destabilizes the immature lattice and promotes formation of the mature capsid lattice. The NC-SP2 and SP2-p6 cleavages are also required for infectivity, as is cleavage of the longer Gag-Pol polyprotein, which liberates the viral enzymes. The mature conical capsid is a fullerene cone, with a curved hexagonal lattice comprising CA hexamers, and the cone ends closed through the incorporation of 12 CA pentamers. CA hexamers are stabilized by binding IP_6 (Dick *et al.*, 2018; Mallery *et al.*, 2018), and differential placement of the hexamers and pentamers produces a variety of related capsid structures that each differ slightly in length and shape (Sundquist and Krausslich, 2012; Mattei *et al.*, 2016; Freed, 2015).

Viral glycoproteins and their fusion peptides that enable entry into target cells must also typically be proteolytically processed to be functional. For example, the HIV-1 Env glycoprotein is synthesized as a polyprotein precursor (gp160), which is inserted into the endoplasmic reticulum membrane co-translationally. Env is glycosylated and then proteolytically cleaved by the host Golgi-associated protease furin as it traffics through the secretory pathway, producing the mature surface gp120 and transmembrane gp41 glycoprotein subunits, which remain non-covalently associated as heterotrimeric spikes. Proteolytic processing exposes the fusion peptide at the gp41 N-terminus and is required for fusogenic activity (Checkley *et al.*, 2011).

In other viruses, proteolytic activation of viral fusion proteins can occur following entry into the target cell. Activation of the EBOV glycoprotein is a particularly well-understood case. EBOV particles associate with the host cell surface by interactions with host receptors that bind to glycans on the viral glycoprotein GP and phosphatidylserine in the viral envelope. After internalization through macropinocytosis, endosomal cysteine proteases such as cathepsins L and B proteolytically process GP to remove a mucin-like subdomain and the glycan cap and expose the receptor-binding site (RBS). The RBS binds the late endosomal/lysosomal protein NPC1, which induces a conformational change in GP, insertion of a fusion loop into the endosomal membrane, fusion of viral and endosomal membranes and release of the nucleocapsid into the cytoplasm (Lee and Saphire, 2009; Carette *et al.*, 2011; Cote *et al.*, 2011; Gong *et al.*, 2016; Wang *et al.*, 2016).

Cell-to-Cell Transmission

After budding, viruses can spread in two different ways; through cell-free transmission and cell-to-cell transmission. Cell-free virions diffuse freely through the extracellular space, and even between organisms, before entering target cells. This process can promote dissemination over long distances, to new tissues, and between hosts. However, untargeted diffusion through aqueous media is relatively inefficient, and free viruses are susceptible to immune recognition. In contrast, viral spread through direct sites of cell-to-cell contact increases transmission efficiency and can help evade antibody recognition. To promote cell-to-cell transmission, viruses often subvert cellular structures that are normally used for cell-cell communication or cargo transfer.

Retroviruses such as HIV-1 and MLV actively promote the formation of adhesive structures between donor and target cells. These stable contact sites are termed virological synapses because they resemble the immunological synapses that mediate antigen presentation, and even employ some of the same molecular components. Virological synapse formation requires interactions between the viral glycoprotein on the donor cell and its cognate target cell receptor. Coreceptors and adhesion molecules are then recruited to stabilize and further organize these contact sites, and the producer cell cytoskeleton is repolarized towards the synapse to promote directional viral assembly and release. Virions bud directly into the intersynaptic space and are transferred efficiently to the closely opposed target cell (Agosto *et al.*, 2015; Bracq *et al.*, 2018; Dufloo *et al.*, 2018; Nejmeddine and Bangham, 2010).

Other viruses hijack existing cell-cell contacts for transmission. For example, neurotropic viruses such as herpesviruses and rabies viruses are transported along axons and spread across synaptic contacts (Koyuncu *et al.*, 2013). Viruses can also achieve targeted release by exploiting membrane protrusions such as nanotubes and filopodia, which normally transmit information and cargoes between cells (Agosto *et al.*, 2015; Bracq *et al.*, 2018; Dufloo *et al.*, 2018).

Conclusions

The principles of enveloped virus budding are remarkably conserved between different virus families, presumably owing to evolutionary history and common functional requirements. Viral egress is typically orchestrated by multifunctional structural proteins that recruit components, assemble the virion, bend host membranes, and facilitate membrane fission. In many, but not all cases, the cellular ESCRT pathway is recruited to mediate the final membrane fission step. Recent studies have also revealed that many traditional "non-enveloped" viruses can be released within vesicles as quasi-enveloped viruses and that viruses frequently alter cellular pathways to promote directional release and synapse formation.

Although the general strategies for enveloped virus egress are increasingly well understood, important challenges remain, including characterizing the release mechanisms of ESCRT-independent viruses, the biology and entry mechanisms of quasi-enveloped viruses, and the molecular mechanisms and pathogenesis associated with cell-to-cell viral spread. These and other

advances will help reveal the best approaches for inhibiting virus release for therapeutic benefit and harnessing release activities in new systems that can be used to deliver biomolecular cargoes into target cells in vivo.

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